THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED JUL 3 1975 Date

Application for Research Grant

(Use extra pages as needed)

June 30, 1975

1. Principal Investigator (give title and degrees):

Herbert B. Herscowitz, Ph.D.
Assistant Professor of Microbiology

2. Institution & address:

Schools of Medicine and Dentistry
Georgetown University
3900 Reservoir Road, N.W.
Washington, D.C. 20007

3. Department(s) where research will be done or collaboration provided:

Department of Microbiology
Department of Pathology

4. Short title of study:

"Effect of cigarette smoke exposure on developmental, cellular and molecular aspects of the immune response."

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

The adaptive immune response has evolved to an extremely refined state in the mammal where it serves a protective function against invasion by potentially pathogenic microorganisms and possibly even more important, to perform a surveillance function by keeping continuously arising malignant cell clones under control. Immunological defects, whether congenital or acquired, result in a susceptible host. The lung of the healthy individual is constantly being exposed to potential insult, which is handled successfully. One such environmental insult on the lung is cigarette smoke. Chronic exposure to cigarette smoke or its components may result in an altered immune response which will ultimately affect the host's health.

The major objective of this research proposal is to further our understanding of the effects of cigarette smoke on the immune response. We will determine the effects of acute and chronic smoke exposure on the functional capabilities of the three principal cell types involved in the immune response; B-cells, T-cells and macrophages. It is becoming increasingly apparent that adult disease is the culmination of a lifetime of insult. In this regard we will study the effects of cigarette smoke on maturational aspects of the immune response in an attempt to define factors which may identify a population at risk to increased frequency of infection or malignancy.

1003545769

Source: https://www.industrydocuments.ucsf.edu/docs/fydm0000

8. Brief statement of working hypothesis:

It is postulated that the immune system serves a surveillance function for control of proliferation of malignant cell clones. Previous studies have shown that patients displaying an altered immune response, either congenital or acquired, are subject to the development of frequent infections and to various forms of malignancy. The alveolar macrophage plays a very significant role in host defense against foreign materials. Macrophages appear to be involved in both the efferent and afferent arms of the immune response to certain antigens, those complex materials which also require T-cell cooperation. It has been demonstrated that interference with macrophage function results in suppression of immune responsiveness. Our previous studies (see Progress Report #3) have shown that cigarette smoke inhibits humoral immune responses. The present studies will delineate the cellular and molecular mechanisms of the effects of cigarette smoke on the immune response.

9. Details of experimental design and procedures (append extra pages as necessary)

See attached Research Plan.

1003545720

Source: https://www.industrydocuments.ucsf.edu/docs/hydm0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Laboratory space currently available to the Principal Investigator consists of one laboratory of approximately 750 sq. ft., including an office, a post-doctoral laboratory of approximately 300 sq. ft., a small animal room of 200 sq. ft. and temporary space in a presently unused laboratory for operation of the Walton Mark II Smoke Machine. My laboratory is equipped with refrigerated centrifuges, automatic gamma spectrometer, fraction collectors, pH meter, spectrophotometer, CO_2 incubator, phase contrast and fluorescence microscopes, electrophoresis equipment, columns for chromatography, facilities for handling of radioisotopes, glassware and reagents. Within the Department of Microbiology are a photographic darkroom, cold and warm rooms, media preparation and dishwashing facilities, amino acid analyzer, recording spectrophotometer, ultracentrifuges, electron microscope. Also available to me through participation in the Pulmonary Program Project is a scanning electron microscope. The Department of Pathology is well-equipped to carry out the processing of tissue specimens for microscopic examination.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

See attached.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See attached.

1003545771

Source: https://www.industrydocuments.ucsf.edu/docs/fydh0000

14. First year budget:

A. Salaries (give names or state "to be recruited")

	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Herbert B. Herscowitz, Ph.D.	50%	$4,431 + 817* = $5,248
Max Robinowitz, M.D.	10%	- 0 -
Technical		
Ruth B. Cooper	100%	$11,000 + 2,200* = $13,200
To be recruited - 1/2 time lab aide (dishwasher, animal caretaker)	50%	3,000 + 600* = 3,600

* Fringe Benefits

Sub-Total for A $ 22,048

B. Consumable supplies (by major categories)

1. Glassware	1,000
2. Disposables (plasticware for culture)	2,500
3. Tissue culture media	2,000
4. Chemicals, isotopes, assay materials	1,500
5. Animals (rabbits and mice)	2,500
6. Miscellaneous	500
Sub-Total for B	$ 10,000

C. Other expenses (itemize)

1. Animal care	4,000
2. Travel	500
3. Publication	500
4. Equipment maintenance (service policies)	800
5. Pathology service	1,000
Sub-Total for C	$ 6,800
Running Total of A + B + C	$ 38,848

D. Permanent equipment (itemize)

Miscellaneous small lab replacement	2,000
Sub-Total for D	$ 2,000
E	5,827
Total request	$ 46,675

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	24,000	9,000	7,000	1,000	6,000	47,000
Year 3	26,400	10,000	7,000	1,000	6,510	50,910

1003545772

Source: https://www.industrydocuments.ucsf.edu/docs/rydm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1. Immunological Function of Alveolar Macrophages	NIH-HL/AI-16748-02	$43,792 (Direct)	May, 1975-April, 1976
2. Preparation of Antibodies Against Prostaglandins	Coleman Associates Contract	1,600	February, 1975-September, 1975

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal Investigator

Typed Name Herbert B. Herscowitz

Signature Herbert B. Herscowitz Date June 30, 197

Telephone 202 (Area Code) 625-7602 (Number) (Extension)

Responsible officer of institution

Typed Name Sam A. Kimble

Title Administrator of Sponsored Programs

Signature Sam A. Kimble Date June 30, 197

Telephone 202 (Area Code) 625-3151 (Number) (Extension)

Checks payable to

Mr. George R. Houston, Jr., Treasurer

Mailing address for checks

Georgetown University
37th and O Streets, N.W.
Washington, D.C. 20057

1003545773

Source: https://www.industrydocuments.ucsf.edu/docs/hydm0000

Budget Justification

1. Duration, Reason for Proposed Period of Support

Support is requested for a three year study. The first year will be devoted to a continuation of the studies of the effect of chronic smoke exposure on immune function. The second and third years will study the cellular and molecular mechanisms along with developmental aspects of immune response.

2. First Year Budget

a. Salaries. In accordance with the policy of Georgetown University, I am requesting that 20% of my salary be paid from this grant. A full-time laboratory technician will be required to participate in this program. I am requesting funds for a 1/2 time laboratory aide to help in the preparation of glassware, sterilization and care of mice used in these studies. There is presently such a person working in my laboratory and being paid from other funds. However, due to expanded effort, the work is too much for only 1/2 time employment. Therefore, contributions from both grants will enable us to have full-time assistance. Also included in these figures are fringe benefits set by the University at 16.7% and 20.0% for 1974-1975 and 1975-1976, respectively.

b. Supplies. These amounts are self-explanatory.

c. Animals. The quality of experimental animals determines the high budget request. In addition, animal costs have been routinely increased, with 10% service charges added on by the Vivarium.

d. Animal Care. The University Animal Care Facility provides us with quarters and care by trained personnel. The costs of these services has risen tremendously over the past years and are scheduled to be increased as of July 1, 1975 again.

e. Travel. Funds are requested for the Principal Investigator to attend one meeting each year which will have relevance to the proposed research. This meeting is usually the FASEB meeting of the American Association of Immunologists which will be held in California next year.

f. Pathology Service. Funds in the amount of $1,000 are requested to cover expenses of Dr. Robinowitz for the pathological examination of the lung material. This amount includes the cost of chemicals, reagents, stains, slides and preparation.

3. Budget for Future Years

1003545774

a. Salaries. We are planning to retain the same personnel with an anticipated 10% increase in salary which was proposed by University regulations.

b. Other Items. There should be no changes in these amounts which are necessary to meet the inflationary rise of scientific supplies.

Source: https://www.industrydocuments.ucsf.edu/docs/rydm0000

Research Plan

A. Background:

It is the mammalian lung that effects the transfer of oxygen by exposing almost the entire volume of circulating blood to the environment. Whereas the relationship between internal and external environments supplies the oxygen necessary for an aerobic existence, it also renders the lung vulnerable to insult by materials carried in ambient air. There is an increasingly expanding body of convincing scientific evidence which suggests that most, if not all pulmonary disease can be related to the inhalation of infectious agents or irritating particulate matter. However, there exists a tremendous gap between scientific knowledge and public attitude in the case of cigarette smoking. While there is as of yet no hard and firm evidence to establish a direct causal relationship, there has been an almost epidemic increase in carcinoma of the lung and chronic obstructive lung disease associated with increased cigarette smoking. There have been numerous retrospective and prospective studies that have shown that the risk of a smoker dying from lung cancer to be as high as 14 times that of a nonsmoker. Cigarette smoking is also closely related to the clinical syndromy commonly grouped together as chronic obstructive lung disease. One such clinical disease, emphysema, is characterized by destruction of alveolar tissue with enlargement of air space distal to the terminal bronchiole, resulting in progressive shortness of breath. Another condition, chronic bronchitis, characterized by hypertrophy of bronchial mucous glands which leads to production of excessive mucus resulting in a chronic productive cough. Carcinoma of the lung and chronic obstructive lung disease are clinically distinct entities, however, they may represent the final stage of continuous irritation.

The effect of cigarette smoking on the human lung was identified during routine autopsy studies performed on healthy young victims of sudden and unexpected death. Niewoehner et al. (1) studied the lungs of 39 such individuals who averaged 25 years of age. Nineteen of the 39 were smokers and 12 of the 19 had either productive coughs or frequent colds. Of the 20 nonsmokers, only 3 had evidence of any respiratory symptoms. Loss of bronchial epithelium and evidence of bronchial wall inflammation were common in the smokers. The most striking abnormality was respiratory bronchiolitis, a lesion of the small airways found in all of the smokers, but also in 5 of the nonsmokers. Clusters of brown pigment-containing macrophages were found in the first and second respiratory bronchioles; edema, fibrosis, and epithelial hyperplasia were seen in the thin walls of these small airways.

There is some evidence which supports the belief that the irritant potential of cigarette smoke first manifests itself as bronchitis and emphysema and ultimately leads to neoplasia. The early work of Auerbach et al. (2) in a detailed blind study of the tracheobronchial epithelium of 456 men and 302 women showed that loss of cilia, basal cell hyperplasia and appearance of cells with atypical nuclei were more common in smokers and increased in proportion to the number of cigarettes smoked. Extensive atypical changes were commonly seen in patients smoking more than 2 packs of cigarettes per day. They also found in one patient, a squamous cell carcinoma in one area together with multiple areas of atypical basal cell hyperplasia at a distance from the carcinoma. However, one should not rule out the possibility that different components of cigarette smoke are responsible for the inflammatory and neoplastic changes observed.

Source: https://www.industrydocuments.ucsf.edu/docs/hydm0000

The mechanisms by which the lung defends itself against the effects of infectious agents, toxic substances, and allergens are complex and still incompletely understood. There is evidence that both humoral and cell-mediated immune responses play a protective role in the lung (3-8). Immunologic damage also occurs in the lung. This is associated with the host's inflammatory response to antigenic material from microorganisms or to proteinaceous material emanating from insects or animal matter, such as pigeon excretia, animal dust, etc., or to reactive chemicals which may function as haptens.

Since any one segment of the respiratory tract differs from others in terms of vascularization, lymphatic drainage, distribution of lymphoid tissue, availability of macrophages, and microanatomy of the mucous membrane, it can be anticipated that the site of deposition of immunogenic material in the respiratory tract will be one of the major factors determining the qualitative and quantitative aspects of the subsequent immune response, and that the site will also be of major pathogenetic significance in the development of immunopathological diseases of the respiratory tract. Creasia et al. (9) showed that upon intratracheal injection of particulate material into small rodents the primary site of deposition was the distal lung. Nettesheim and Williams (7) employed this observation to determine whether antigens that are allowed to penetrate to the alveolar parenchyma are capable of effectively triggering an immune response and where this immune response is located. Using respiratory-disease-free mice, they showed that particulate antigens (RBC) were ineffective in triggering a humoral immune response when deposited in the deep lung. However, when larger amounts were applied, an immune response was observed and the spleen appeared to be the major location of most of the antibody-forming cells. In addition, the spleen also contained primed memory cells which responded to secondary antigenic stimulus. This was shown by experiments in which either the numbers of antibody-forming cells were enumerated in the lungs, lymph nodes and spleens of intratracheally-exposed animals or by experiments where the spleen was removed at various times before or after antigenic challenge. Their studies also demonstrated that once a primary antibody response had been successfully induced via the respiratory tract, local as well as systemic immunity developed, that is, an anamnestic response could be initiated via the intratracheal, as well as via the intravenous route, accompanied by a massive influx of lymphoid elements into the lungs in both cases. Hiramoto and Hamlin (10) previously showed that antibody-forming cells appeared in the lung during an anamnestic response in which both primary and secondary antigenic challenge were given intravenously. Nettesheim and Williams (7) concluded that nontoxic, particulate antigens deposited in the alveolar parenchyma are readily eliminated or rendered nonimmunogenic and that only a small proportion of the immunogen is capable of entering the blood stream, probably at the level of alveolar capillaries and initiates a systemic immune response. These findings are in contrast to the studies of Holub and Hauser (11) who reported a predominantly local antibody response by lung alveolar histiocytes after intratracheal instillation of antigen and the studies of Johnson and Waldman (3) in which it was shown that local introduction of antigen into the respiratory tract resulted in an optimal local pulmonary response with little or relatively less evidence of systemic responses; the converse was also true. Of further interest were their studies in which mice were exposed to killed Bordetella pertusis organisms or to live Sendai virus prior to intratracheal or intravenous stimulation with the hetero-

1003545726

Source: https://www.industrydocuments.ucsf.edu/docs/rydm0000

logous RBC. They observed a marked inflammatory response as a consequence of the additional microbial agents which was accompanied by an enhanced antibody response to SRBC when the antigen was administered intratracheally. These experiments suggested that respiratory infections might play an important role as "conditioners" or "adjuvants" in the induction of the immune response in the deep lung. Finally, their studies also demonstrated secondary responses for pulmonary cell-mediated immunity which suggests the probability of a capacity for memory by pulmonary T-lymphocytes.

One of the major cell types of the alveolus is the alveolar macrophage. This mononuclear cell is derived from a bone marrow precursor but undergoes differentiation in the lung presumably to adapt to the high oxygen environment of the alveolus. This cell is the principal macrophage of the lung and is responsible for the uptake and removal of inhaled particles (12). Thus it acts to protect the respiratory membrane from the tissue-damaging properties of inhaled particles and also may act as a transport which out of the lung for foreign materials. The intrapulmonary processing of foreign particles deposited on the respiratory membrane depends on the primary and secondary mechanisms directed at the physical, chemical and biological properties of the particle which are associated with its pathogenic potential. Primary mechanisms include the coating actions of secretory materials, chemical interactions with oxidant gases, solubilization of salts and phagocytic ingestion by resident alveolar macrophages. The primary particle processing mechanisms are enhanced by secretory, humoral and cellular-immune mechanisms resulting from previous exposure. Secondary cellular and humoral defense mechanisms are brought into play when the primary systems fails to process adequately the inhaled particle. This complex set of mechanisms provides many opportunities for defective function in particle uptake or intracellular processing phases of phagocytosis, in the subsequent immune responses, and in protective mechanisms against the proteolytic enzymes released in the inflammatory response. For example certain particulate materials such as silica or asbestos may induce direct lysozomal damage, cell death, and inflammation (13). Environmental gases inhibit cell enzymes; cigarette smoke inhibits glyceraldehyde dehydrogenase, anaerobic glycolysis and particle uptake (14). Such environmentally-induced defects in macrophage handling of foreign particles may have their ultimate effect on the ability of the host to mount an immune response.

1003545777

In recent years, a great deal of information has been gathered in relation to the cellular components of the immune response. In mammals, like in avian species, there are two primary lymphoid compartments. The thymus, which has an influence on the cells (T-cell) that participate in cell-mediated immunity and the analogue of the bursa, which has an influence on the cells (B-cells) that synthesize antibody. In addition to their separate functions it has been shown that the ability of a B-cell which has been exposed to antigen to initiate antibody production depends for many, but not all, antigens on the concomitant activity of specifically stimulated T-cells. These T-cells can exert a regulatory influence on several B-cell functions: (a) the switch from IgM to IgG antibody synthesis; (b) selection of clones producing antibody of high affinity; (c) suppression of B-cell function under certain circumstances (15). There is also an impressive body of literature which implicates a third cell type in both the efferent and afferent limbs of the immune response. This cell type, referred to as an accessory cell, has enjoyed varing degrees of favor among immunologists in regard to cyclical feelings of its importance in the immune response. There are numerous reviews which have been devoted to the function of macrophages, especially their role in the immune response (16-19).

Source: https://www.industrydocuments.ucsf.edu/docs/lydm0000

By definition, the basic functional property of a macrophage is its ability to engulf foreign materials. Macrophages take up materials essentially by two similar processes, phagocytosis (particulate materials) and pinocytosis (soluble matter). Both processes are initiated by the interaction of the foreign material with the cell membrane (20). The material, after entry into the cell, becomes surrounded by a membrane which eventually coalesces to form the phagosome. The phagosome moves to the interior of the cell where it subsequently fuses with a preformed lysosome to form a phagolysosome (21). The hydrolytic enzymes of this secondary lysosome catabolize the ingested material and for many substances, the endocytic function of the macrophage is fulfilled. It is this product of endocytosis that has intrigued immunologists, for this may be the initial stage of antibody formation.

The relevance of phagocytosis to immune processes of host protection is still somewhat unclear. While some suggest that there is no correlation between susceptibility to phagocytosis and immunogenicity (22) others have shown that some of the phagocytized antigen remains associated with the macrophage surface and is more immunogenic than free antigen alone (23). Evidence for the role of the macrophage in humoral responses was provided by numerous in vivo studies using irradiated mice (24), reticuloendothelial blockade (25) and anti-macrophage serum (26-28). The essentiality of macrophage participation in the primary in vitro immune response was shown by Mosier (29). In mouse spleen cultures, antibody production appeared to involve both the phagocytosis of antigen by a cell population capable of adhering to glass (macrophage-rich) and antibody production by a non-adherent cell population (lymphocyte-rich). Later, the non-adherent cell population was further resolved into its T and B cell components (30). Several possible functions have been assigned to the macrophage in its participation in the immune response: (a) antigen processing; (b) antigen presentation due to surface-bound components; (c) providing the necessary micro-environment for lymphoid cell survival; (d) mediation of the signal for antibody production by B-cells. An intriguing model of antibody formation recently proposed by Feldmann (31) employing the three functional cell types does not preclude any of the above mentioned roles for the macrophage.

The literature discussed above comprises only a small proportion of that which exists regarding the role of the macrophage in the induction of the humoral immune response. Further, the literature regarding the role of the macrophage in cell-mediated immunity is almost as voluminous and has recently been reviewed by Nelson (32). It soon becomes apparent that there is no consistent agreement among investigators as to the precise role of macrophages in immune responses and a great deal is yet to be done to further our understanding. **1003545778**

To return to the main stream of purpose, the alveolar macrophage system constitutes an important cellular defense mechanism in the lung, by virtue of its role in removing foreign substances. It is also possible that these cells co-operate with lymphocytes in situ in the lung or transfer antigen to lymphocytes in regional lymphoid organs. Consequently, any environmental factors that interfere with the function of alveolar macrophages may be considered to be of potential danger to the immune capabilities of the host. In recent years there has been a significant amount of interest in the effect of tobacco smoke on macrophage function. The exposure of alveolar macrophages to tobacco smoke or its components has been shown to induce a number of metabolic and functional changes in these cells. Alterations have been demonstrated in the resting rate of glucose oxidation (33), protein synthesis (34), the activity of various enzymes (35-36), ultrastructure (33,37), cell growth and nucleic acid synthesis in vitro

Source: https://www.industrydocuments.ucsf.edu/docs/hydm0000

(38-39), and phagocytic ability (40-41). In addition, recent reports suggest that cigarette smoke or components thereof have an adverse effect on the immune response. Holt and Keast (42) studying the acute and chronic effects of cigarette smoke on mice have demonstrated significant death of cells of the immune series both in vivo and in vitro following acute smoke exposure. Chronic exposure to smoke on the other hand resulted in increased numbers of macrophages and lymphocytes in the lungs of experimental animals. Finklea et al. (43) have shown that young adult cigarette smokers had significantly lower concentrations of antibody to influenza virus A_2 after either natural infection or vaccination as compared to a similar group of nonsmokers. Roszman and Rogers (44) demonstrated that there was a dose-dependent suppression of the in vitro anamnestic response of rabbit lymph node cells to SRBC by nicotine and the water-soluble fraction of cigarette smoke. Suppression could be achieved after exposure of the cultures to either agent for a period of 2 hours before antigenic challenge, thereby suggesting that these materials induce a rapid and irreversible effect on lymphoid cells. Using the C57 Bl mouse as a model, Thomas et al. (45) showed that mice receiving 26 weeks of exposure to cigarette smoke had decreased primary and secondary immune responses in all organs examined after intratracheal instillation of SRBC. The primary response in the lung was completely suppressed whereas the secondary response was delayed. Primary responses in mediastinal and cervical lymph nodes and spleen were short-lived while secondary responses in these organs were delayed. In an additional study, the same authors (46) exposed mice to cigarette smoke for up to 42 weeks. In mice exposed to smoke only during the week following immunization, the response was not impaired in any of the lymphoid organs examined. However, a decrease in the primary response in the lungs was observed in mice exposed to smoke for 9 days prior to antigen administration. The responses found in spleen during the initial 120 days of exposure were erratic. However, after 294 days of exposure the response in spleen was depressed in all animals. The lymph node response was consistently first enhanced and then depressed by cigarette smoke exposure. Esber et al. (47) showed that inhalation of whole cigarette smoke or its vapor phase impaired the hemagglutinin response of mice injected with SRBC. An immunosuppressive effect was observed in animals exposed to whole smoke or vapor phase for seven days before or two days after administration of antigen. This effect was apparently temporary since there was recovery of immune responsiveness about 14 days after exposure. It was further found that the early or inductive phase of both primary and secondary responses rather than the late or productive phase was affected by cigarette smoke. This suggests that the suppressive effect of cigarette smoke may be functioning at the level of the macrophage. A similar recovery from the immunosuppressive effects of cigarette smoke has been reported by others (48). In an additional study, Thomas et al. (49) showed that there were only minor differences in immune responses of animals exposed to whole smoke and challenged with antigen by a systemic route rather than intratracheally. This appears to be in opposition to the results of Esber et al. (47) described above.

1003545779

Studies of the effects of cigarette smoke on cellular immune function have also recently appeared. Rylander (50) showed that guinea pigs used in four and eight-week inhalation experiments had increased numbers of macrophages and leukocytes in the lung. Smoke that had been passed through Cambridge filter did not cause the effects. Thomas et al. (51) exposed mice daily to fresh cigarette smoke for up to 35 weeks and evaluated cell-mediated immunity as measured by Phytohemagglutinin responsiveness of lymphocytes from various organs. They observed an initial enhancement of PHA responsiveness in lymphocytes from regional lymph

Source: https://www.industrydocuments.ucsf.edu/docs/nyam0000

nodes and peripheral blood but by the 35th week of exposure the response was markedly suppressed. On the other hand, Suciu-Foca et al. (52) and Whitehead et al. (53) demonstrated that there were no differences in PHA responsiveness in lymphocytes obtained from smokers compared to age-matched non-smoking controls. The latter study also showed that the addition of nicotine to cultures of peripheral human lymphocytes had no effect on PHA-responsiveness. These studies are in contrast to the study of Desplaces et al. (54) who found tobacco smoke inhibited lymphocyte by PHA but stimulated lymphocytes in the absence of PHA. The studies of Warr and Martin (55) showed that MIF reduced the migration of alveolar macrophages from nonsmokers but was ineffective in preventing migration of alveolar macrophages from smokers. Migration of macrophages from nonsmokers with delayed type hypersensitivity skin reactions to Candida was inhibited when antigen was present in the medium. Antigen did not inhibit macrophages who lacked delayed hypersensitivity to the antigen or macrophages from cigarette smokers. Finally, Roszman et al. (56) showed that nicotine or water soluble fraction of whole cigarette smoke inhibited transformation of rabbit peripheral blood lymphocytes stimulated with anti-rabbit Fab (B cell mitogen) and concanavalin A (a T cell mitogen). These results deserve further investigation in terms of the role of macrophages in these responses.

Although the above described studies represent a vast refinement of experimental design over those previously reported it is quite obvious that there is a lack of agreement of experimental results. Whereas one group demonstrates an irreversible effect of cigarette smoke, others point toward reversibility. Whereas some find a suppressive effect on systemic immunity, others limit the effect to the lung. Where some restrict the inhibitory effect to particulate matter in whole smoke others find it with the gas phase. Whereas some find an effect on cell-mediated immune function others do not. All of these differences may be due either in total or in part to the differences in cigarettes used, experimental animal, state of health of animal and conditions used for smoke exposure. In order to generate meaningful results these conditions must be cautiously defined and clearly stated so that results can be compared.

B. Rationale and Working Hypothesis:

It is generally accepted that the adaptive immune response evolved as a general vertebrate protective mechanism to eliminate "non-self" components (57). Burnet suggested that the immune system functions as a surveillance mechanisms to aid the individual against life-threatening challenge. The surveillance concept has been suggested as a defense in human cancer. In simplest terms, surveillance assumes that tumor cell clones emerge continuously during the life of an individual, and that effective mechanisms exist by which the foreigness (tumor clone) is recognized, attacked and destroyed. This can be explained on an immunological basis. There is sufficient evidence to indicate that in the process of transformation to a neoplastic condition, the cell acquires which were not present on the normal parent cell, thus the cell is no longer recognized as self and as a consequence is rendered susceptible to immune attack on the basis of its newly recognized antigenic makeup. One prediction of this theory is that individuals who have immune deficiency, either congenital or acquired, should be subject to a higher incidence of cancer. Indeed, the evidence of Good and Finstad (58) indicates that patients who have congenital immunological defects and patients who have been immunosuppressed following organ

1003545780

Source: https://www.industrydocuments.ucsf.edu/docs/ydmo0000

transplant develop neoplasms with a greatly increased frequency. Further, in man, the incidence of cancer greatly increases with ensuing age. It has thus been suggested that the development of cancer is associated with a progressive inability to eliminate foreigness which is related to a decrease in efficiency of the immunological defense mechanisms (59). It should be noted that most chemical carcinogens are also immunosuppressive (60) and that many, if not most, viruses known to cause cancer in animals can also suppress the host's immunological reactivity. Thus it would appear that any alteration of the immunological defense mechanism may contribute to the establishment of malignant cells and the eventual demise of the host.

It is apparent that the cells of the reticuloendothelial system are actively involved in maintaining the health of the individual. Numerous studies suggest that macrophages are involved in the induction of both the humoral and cell mediated immune response, probably at the level of antigen processing. There is evidence to indicate that blockade of RES function results in an inhibition of immune responsiveness. Several studies have shown that tobacco products interfere with the phagocytic capabilities of the macrophage as well as with the host's immune response. This research proposal is designed to determine the cellular and molecular mechanisms, involved in the immunosuppressive effect of cigarette smoke.

C. Specific Aims:

The specific aims of this research proposal are to obtain an understanding of the effects of cigarette smoke on the immune response. We are interested in determining the cell type affected and the molecular mechanisms involved. We will study the effects of cigarette smoke on the maturation of the immune response to determine potential populations at risk to increased infection or malignancy. Experiments will be devised to elaborate and evaluate the following parameters:

Year 1

1. To further define the immunosuppressive effect of cigarette smoke on the immune response of mice.
   a. To study the effect of chronic smoke exposure on humoral antibody production.
      (1) To compare Kentucky Reference cigarettes (1A1 and 1R1) in these studies.
      (2) Evaluate plaque-forming cells and serum antibody levels.
   b. To study the effect of chronic smoke exposure on correlates of cell-mediated immunity.
   c. To evaluate the immunosuppressive effect of the vapor phase of cigarette smoke on humoral and cell-mediated immunity.
      (1) To compare 1A1 and 1R1 cigarettes
      (2) To compare acute and chronic exposure to smoke.

Year 2

1. To determine the effect of cigarette smoke on the individual cell types involved in the immune response.
   a. To study the effect of acute and chronic smoke exposure on macrophage function.
      (1) To determine the effect of *in vitro* and *in vivo* smoke exposure on phagocytic and antigen-processing capabilities.
   b. To study the effect of acute and chronic smoke exposure on B-cell function in the absence of T cell regulatory effects.

1003545781

Source: https://www.industrydocuments.ucsf.edu/docs/fydm0000

(1) To employ the congenitally athymic mouse in studies of immune function.

c. To study the effect of smoke exposure on T-cell function.

d. To determine the effect of cigarette smoke on macromolecular synthesis in B cell, T cells and macrophages.

Year 3

1. To determine the effect of cigarette smoke on developmental aspects of the immune response.

a. To study the long term effects of cigarette smoking of pregnant mice on the immune function of their progeny.

b. To study the effects of cigarette smoke on immune function of animals of different ages.

(1) To determine if neonatal exposure to cigarette smoke has any long term effects on the quality or quantity of immune responsiveness.

All throughout these studies, the lungs from mice exposed to the various smoke exposure protocols will be supplied to Dr. M. Robinowitz of the Pathology Department who will evaluate them for intercurrent microbial infection and for smoke-induced pathological changes. Having materials from animals involved in clearly defined and controlled smoke exposure protocols will enhance our understanding of potential pathology induced by cigarette smoke.

D. Experimental Design and Procedures:

1. General Outline

It is becoming increasingly clear that requirement for collaboration among different cell types in immune responses is a function of the physical and chemical structure of the antigen. The chemically complex materials, that is, those substances containing a small number of diverse antigenic determinants appear to require the collaborative interaction of T and B cells for antibody production. On the other hand, those antigens which contain a large number of repetitive chemical structures appear to stimulate B cells directly in the absence of T cell help. In a like manner, the requirement for macrophage participation in immune responses also appears to be based on antigen structure. The complex, particulate antigens appear to require some interaction with macrophages, either for processing or presentation to the appropriate immunocompetent cell type. The less complex materials appear to be independent of macrophage handling. Thus one could speculate that the T-dependent antigens also require macrophage participation in the initiation of immune responses, whereas T-independent antigens are also macrophage-independent.

Our previous results (see Progress Report #3) have shown that acute exposure of mice to smoke from Kentucky Reference 1R1 cigarettes caused marked suppression of the appearance of plaque forming cells in the spleen. Our working hypothesis is based on convincing evidence which suggests that macrophages contribute to the initiation of immune responses at the level of antigen processing to many, but not all antigens. Further, it has been suggested that interference with the phagocytic function of the macrophage results in an altered immune response. Therefore, based on this evidence the most likely target cell for the immunosuppressive effect of cigarette smoke would be the macrophage. Although this cell type has been implicated there has been no rigorous demonstration of the interference. One of our major goals therefore will be to determine the effects of cigarette smoke at the cellular and molecular level. Our laboratory

1003545782

Source: https://www.industrydocuments.ucsf.edu/docs/rydm0000

has the animal systems, techniques and reagents available to undertake such a study. For example, our anti-macrophage serum can be used to obtain populations of B and T cells devoid of macrophages, whereas our anti-plasma cell serum can be used to obtain populations of T cells and macrophages. Using these reagents in combination in the athymic mouse system we can obtain "pure" populations of B cells and macrophages. One test of the hypothesis that T-independent antigens are also macrophage independent and a possible means of determining the cells affected would be to employ nude mice in smoke exposure protocols. At this time, I would predict that such animals would be refractory to the immunosuppressive effects of cigarette smoke if macrophages were the target cells and were not required for the immune response to T-independent antigens. We can also use these isolated cell types to study the in vivo and in vitro effects of cigarette smoke on response to mitogens and phagocytic capabilities.

Another major emphasis of this proposal is directed toward an understanding of the effects of cigarette smoke on developmental aspects of the immune response. Newborn mice fail to produce antibodies to SRBC (61) and newborn rats will accept allogenic skin grafts (62). Newborn rats have been shown to lack the follicular and medullary antigen-trapping structure characteristic of adult animals. Further, there is an apparent absence of functional macrophages in newborn animals. Moreover, the transfer of macrophages from adult donors into newborn animals has resulted in an increased immune response thereby suggesting that immunological immaturity may be due, at least in part, to an inability of the neonate to handle antigens, thus ascribing this defect to immaturity of macrophages (61,63-64). As part of our Pulmonary Program Project, my laboratory is interested in the immunological functions of alveolar macrophages, specifically their ability to serve an accessory function in humoral responses. One aspect that will be studied in collaboration with Dr. J. Bellanti is the maturation of immunological function of alveolar macrophages. To extend this further, the present protocol will study the effects of cigarette smoke on the maturation of immune responsiveness. We are interested in determining the effect of cigarette smoke on the immune responsiveness of progeny obtained from smoked-pregnant mice.

2. General Procedures

a. Animals

Two animal systems will be used in these studies. Rabbits will be used for studies of the in vitro anamnestic response and as a source of alveolar macrophages for the following reasons: the lung is the prime target of exposure to tobacco products, the rabbit is of sufficient size to yield a workable number of cells, and little is known concerning the contribution of alveolar macrophages to the immune response. Balb/c mice will be used for continued in vivo studies of the effect of cigarette smoking on the immune response, in vitro studies, mitogen studies, developmental studies and as a source of macrophages.

(1) Isolation of Macrophages:

The method of Myrvik et al. (65) will be employed to obtain unstimulated rabbit alveolar macrophages. Briefly, rabbits will be sacrificed by injection of a lethal dose of Diabutal (Diamond Labs, Des Moines, Iowa), the chest cavity opened aseptically and the trachea closed by means of a sterile

1003545783

Source: https://www.industrydocuments.ucsf.edu/docs/nyum0000

hemostat. The trachea will be transected above the clamp and the lungs, heart and trachea will be dissected out. A volume of 30 ml. of Hank's balanced salts solution will be injected into the trachea until the lungs are distended, the lungs massaged and the fluid drained into sterile centrifuge tubes. The cells will be recovered by centrifugation at 1,500 rpm for 15 minutes and resuspended in culture medium containing 20% normal rabbit serum. The cell suspension so obtained has been shown to be contaminated by less than 2% of other cell types.

Both stimulated and unstimulated peritoneal macrophages will be obtained from mice. For stimulated peritoneal exudate cells, mice will be injected with 3 ml. of fluid thioglycollate medium (Difco Labs, Detroit, Mich.), according to the procedure of Argyris (66). Three days later, the mice will be sacrificed by exposure to chloroform vapor and 3 ml. of BSS containing 30 units of heparin will be injected into the peritoneal cavity. After gentle massage of the peritoneum, the fluid will be collected and cells recovered by centrifugation as above. The cell pellet will be resuspended in culture medium containing 20% of new-born calf serum. Unstimulated peritoneal cells will be collected by a similar procedure except that fluid thioglycollate will not be injected. Alveolar macrophages will also be obtained from mice by a modification of the Myrvik procedure, or as described by Bennett (67).

b. Antigens

Keyhole limpet hemocyanin (KLH) which is a potent immunogen, inducing high concentrations of antibody, evoking high levels of immunological memory, and involving a large number of cells in the synthesis of antibody (68) will be used as the antigen. KLH obtained as an ammonium sulfate slurry (Pacific Bio-Marine Supply Co., Venice, Calif.) will be purified according to the procedure developed in our laboratory (69). Briefly, after extensive dialysis, the material will be concentrated by ultrafiltration using an XM-100 membrane (Amicon Corp., Lexington, Mass.). The KLH will be fractionated on a column of DEAE-cellulose employing a linear salt gradient of 0.05 M NaCl - 0.05 M PO_4, pH 6.7 to 0.4 M NaCl - 0.05 M NaCl - 0.05 M PO_4, pH 6.7. The first peak eluted from the column will be concentrated by ultrafiltration, dialyzed and fractionated on a column of Bio-gel A 5 m (Bio-Rad Labs, Richmond, Calif.). The first peak, which is the purified KLH, shows a single component as determined by the following criteria: double diffusion in agar, immunoelectrophoresis, analytical ultracentrifugation, and disc electrophoresis. This material will be adjusted to a final concentration of 2 mg/ml, sterilized by Millipore filtration and stored at 4°C until used.

Sheep erythrocytes will be obtained from Microbiological Associates, Besthesda, Md., as a 20% suspension in Alsever's solution. The cells will be washed 3 times in cold phosphate buffered saline, pH 7.2, and resuspended in the same buffer at the appropriate concentration.

The thymus-independent antigens, dextran isolated from Leuconostoe mesenteroides B-1355 as described by Proctor (70) and lipopolysaccharide (Difco Labs, Detroit, Mich.) will also be used in these studies.

c. Tissue culture methods

(1) In vitro primary immune responses

Spleen-cell cultures will be prepared from inbred mouse strains

Source: https://www.industrydocuments.ucsf.edu/docs/rydm0000

as described by Mishell and Dutton (71). Cell suspensions will be diluted to the desired concentration in monolayer type MEM containing Hank's salts supplemented with sodium pyruvate, amino acids, vitamins, glutamine and 10% fetal calf serum, all obtained from Grand Island Biological Co., Grand Island, N.Y. The cultures will be distributed in Petri dishes at varying cell concentrations and incubated at 37°C in an atmosphere of 10% CO_2 and 83% N_2 on a rocker platform. The culture dishes will receive supplemental nutritional mixture and fetal calf serum daily as described (71).

(2) In vitro secondary response

Lymph nodes or spleens from rabbits previously primed with KLH in vivo will be surgically removed, trimmed of connective tissue and weighed. Cell suspensions will be prepared by disrupting the organ with stainless steel screens. Cells will be washed in 0.15 M PBS, pH 7.2, and adjusted to a final concentration of 2×10^7 nucleated cells/ml. Culture will be carried out in MEM as we have described previously (72). To detect newly synthesized antibody, ^{14}C-leucine will be added to the culture medium and cells incubated at 37°C on a roller drum.

A portion of the cell suspensions will be stimulated with 100 μg of KLH while another portion will be incubated with BSS and will serve as control. After 24 hours, the antigen will be removed from cultures by sedimentation of the cells followed by washing to remove unbound antigen. The culture medium will be changed completely on alternate days. For the determination of newly synthesized antibody, the extracellular medium will be dialyzed extensively to remove unincorporated ^{14}C-leucine and subjected to the assay procedure described below. For the determination of cells engaged in antibody formation, cell samples will be removed on various days and assayed by the plaque technique which will be described below.

d. Methods for evaluation of immune response

(1) Plaque assays

(a) Sheep erythrocytes

Cells releasing hemolysin for SRBC will be enumerated on the 4th or 5th day of culture by the PFC technique of Jerne and Nordin (73) as modified by Cunningham and Szenberg (74) for use on glass slides. Cells will be scraped from culture dishes and transferred to glass tubes where they will be dispersed. After determination of recovery and cell viability, 0.05 ml. of cells will be mixed with SRBC and complement and transferred to glass microscope slides. Plaques will be developed during a 1-2 hour incubation period at 37°C and counted. A similar procedure will be used for spleen cells from animals used for in vivo protocols.

(b) Keyhole limpet hemocyanin

Cells will be assayed for their ability to form hemolytic plaques against KLH-coupled RBC as described previously (75). Briefly, spleen cells at a concentration of 4×10^6/ml will be mixed with 4×10^8 SRBC/ml which have been conjugated with KLH using chronic chloride. After addition of guinea pig complement the mixtures will be added to each of 3 chambers formed on a glass slide and incubated at 37°C. Plaques will be enumerated by examining the slides under low magnification. To detect indirect plaques (IgG, PFC), an appropriate concentration of anti-IgG facilitating serum will be added to the cell mixture.

(c) Dextran, Lipopolysaccharide

Plaque assays for these antigens will be carried out according to the method of Proctor (70).

1003545785

Source: https://www.industrydocuments.ucsf.edu/docs/xyam0000

(2) Radiolabeled protein and antibody assays

Newly synthesized extracellular proteins will be determined by precipitation of culture fluids with cold 5% TCA as previously described (72). De novo synthesized antibody will be measured by reaction of culture fluids with an insoluble immunoabsorbant as described by Self et al. (76) and modified by Herscowitz and Stylos (77). This method is an extremely sensitive and specific assay of antibody synthesis including antibody of low affinity.

(3) Hemagglutinating antibody

Serum antibody against SRBC will be assayed by standard hemagglutination using the Microtiter system (Cooke Engineering, Alexandria, Va.). Antibody against KLH, dextran or LPS will be measured by passive hemagglutination in the same system using methods described above for preparation of antigen-coated RBC.

e. Mitogen studies

Determination of the optimal concentration of mitogen (Phytohemagglutinin P, Difco Labs.; Concanavalin A, Miles Labs.; Lipopolysaccharide - Salmonella typhosa 0901, Difco Labs.) will be carried out according to the method of Peavy et al. (78). Briefly, 3 x 10^6 cells are incubated in 0.8 ml of complete MEM containing 10% fetal calf serum, 2 mM glutamine, MEM nonessential amino acids, penicillin, streptomycin and fungizone and 37°C under 5% CO_2 in air for 48 hours in the presence of varying concentrations of the mitogen. After this period, 1 μCi of methyl-^{3}H-thymidine will be added and incubation continued for 24 hours. Cells will be harvested by centrifugation, washed in cold 0.15M NaCl, twice in cold 5% TCA and once in cold methanol. The precipitates are then air dried, solubulized for 2 hrs at 45°C in 0.25 ml of Nuclear Chicago Solubulizer and counted in a liquid scintillation detector.

f. Exposure to cigarette smoke

(1) Cigarettes

The cigarettes used in these studies are obtained from the University of Kentucky Tobacco and Health Institute and include the 1A1 (low nicotine) and 1R1 (high nicotine) reference cigarettes. Ten thousand cigarettes from the same lot were purchased and are stored in the original cartons plastic bags at -20°C. One week before use, the cigarettes are removed from the freezer, packages opened and stored in a humidified chamber at room temperature. Cigarettes were weighed before use so that uniformity could be maintained.

(2) Smoke exposure

Mice were exposed to cigarette smoke on the Prototype Mark II Walton Horizontal Smoke Exposure Machine. Routine calibration of the machine is made to achieve uniformity. The rotometer is set at 10. The machine is set to give a 2 sec. puff, 28 sec mix and 30 sec purge to complete the cycle. Exposure consists of the first 8 puffs of the cigarette. Twenty animals are always exposed to cigarette smoke at the same time so that equivalent exposure is given to all animals. Before each experiment the mice are acclimated to the smoking machine by putting them through 8 puff cycles, two times a day for a period of one week. Those animals to be used in smoking experiments are then exposed to increasing numbers of puffs of cigarette smoke for 2 days before they enter a smoking protocol. Sham-smoked and cage-held animals will always be used as controls.

(3) Animals

Male Balb/c mice, weighing between 13-15 grams have been used

Source: https://www.industrydocuments.ucsf.edu/docs/nyfm0000

in the past. These animals were chosen for the following reasons. Although they contain endogenous type A and type C virus particles, spontaneous induction of leukemia is not characteristic of Balb/c mice as with other inbred strains. Secondly, these animals are low inducers of aryl hydrocarbon hydroxylase, an enzyme involved in the metabolism of aromatic hydrocarbons and thought to be related to the carcinogenesis of tobacco products. Every attempt is made to use healthy animals in these experiments. We have had some problems in the past with intestinal parasitic and worm infections in mice and have excluded these animals from the study.

3. Experimental Approach:

a. Effect of chronic exposure to cigarette smoke on humoral immunity

Groups of mice will be exposed to smoke from Kentucky Reference 1A1 and 1R1 cigarettes according to various protocols for up to 40 weeks. Following smoke exposure, the animals will be evaluated for their ability to produce serum antibody and numbers of splenic plaque-forming cells will be determined. The following parameters will be evaluated:

1) type of cigarette - 1A1 vs 1R1
2) number of cigarettes - 1,2, or 3/exposure
3) number of exposures per day - 1,2, or 3
4) duration of exposure - 10,15,20,25,30, etc., weeks
5) effect of withdrawal from smoke protocol on recovery of immune response
6) time of antigen administration
7) type of antigen used - T-dependent vs T-independent

b. Effect of chronic exposure to cigarette smoke on cell-mediated immunity

Unanue and Feldman (79) have shown that injection of macrophage bound antigen can induce a state of delayed hypersensitivity in animals. Alveolar and peritoneal macrophage will be harvested from normal animals and incubated with KLH. After washing the antigen-containing macrophages will be injected alone or in Freund's complete adjuvant into the footpads. Some 2-4 weeks later, the animals will be challenged with soluble antigen injected into the skin. The degree of erythema and induration will be measured some 24-48 hours later. These experiments will compare macrophages from smoke-treated animals and sham-smoked injected into both smoked and sham-smoked recipients.

Warr and Martin (55) have shown that alveolar macrophages from human smokers were insensitive to the action of MIF obtained from healthy volunteers and that the presence of antigen did not inhibit macrophage migration in preparations derived from antigen-sensitive smokers. Experiments will be set up to evaluate similar parameters in smoked-animals. We will determine if alveolar and peritoneal macrophages from such animals are similarly refractive to the activity of MIF generated from healthy animals. In a like manner, we will evaluate the ability of smoked animals to generate MIF to be used in assays with macrophages from normal animals. Pelley et al. (80) showed that MIF was produced in response to KLH stimulation. In order to carry out these experiments, MIF will be generated from lymph nodes of normal and smoked animals according to the procedure of Rocklin et al. (81) as used by Pelley et al. (80) lymph nodes from KLH-stimulated animals will be cultured in vitro in cell sus-

Source: https://www.industrydocuments.ucsf.edu/docs/hydm0000

pension. One portion of the cell suspension will be restimulated with the appropriate concentration of soluble KLH to stimulate production of MIF. Another portion of the cell suspension will be cultured in medium alone to generate control supernatant fluids. Supernatant fluids will be removed and pooled for 3 consecutive days, and cells will be resuspended in fresh tissue culture medium. The control supernatant fluids will be divided into two equal fractions. One fraction will be used unchanged while antigen will be added to a second fraction in the same concentration used for production of MIF. The supernatant pools will be centrifuged, dialyzed, lyophilized and stored at -70°C until used, at which time the sample will be reconstituted and filter sterilized before use. The MIF will be assayed with both peritoneal and alveolar macrophages obtained from smoked and control animals as described by Pelley and Schwartz (82) or by Warr and Martin (55) as adapted for alveolar macrophages.

c. Effect of vapor phase of cigarette smoke on the immune response

In view of our previous findings that 1R1 but not 1A1 cigarettes had an immunosuppressive effect in acute smoke exposure protocols we will test the effect of removing particulate materials from smoke by placing a Cambridge filter in the smoke generating machine. The first factor that must be explained is the role of nicotine in immunosuppression. If immunosuppression is still observed with vapor phase smoke of 1R1 cigarettes we can most likely rule out the effect of the high nicotine content of these cigarettes and attribute the results to differences in other organic constituents. On the other hand if immunosuppression is lost with the vapor phase then nicotine must play some possible role. Experiments will be set up to evaluate the effects of acute and chronic exposure to the vapor phase of cigarette smoke on both humoral and cell-mediated immunity as described in sections (a) and (b) above testing similar parameters.

why only nicotine as suspect?

d. Effect of cigarette smoke on the individual cell types involved in the immune response.

(1) Effect on macrophages.

(a) Phagocytic Function

Animals from which alveolar macrophages are to be obtained will be exposed to whole smoke or its vapor phase for varying periods of time. The animals will be sacrificed and macrophages will be obtained which will be tested for phagocytic function as well as the ability to participate in an immune response.

It was observed by Cohn and Ehrenreich (83) that the pinocytosis of certain disaccharides by mouse peritoneal macrophages leads to extensive vacuolization of the macrophage cytoplasm. The vacuoles were shown to be lysosomes containing the pinocytosed disaccharide. Disaccharides which induce vacuolization were indigestible and evidently too large to escape readily from the macrophage lysosomes. Thus one has a visible marker to determine the extent of phagocytic function of a cell population.

Both macrophages obtained from smoked animals and pure cultures of alveolar and peritoneal macrophages will be prepared by the methods of adherence and density gradient centrifugation. Cultures will be exposed to smoke and/or vapor phase for varying periods of time. Initial experiments will be directed toward the determination of optimal concentration and duration of exposure to tobacco products which will allow for viable macrophage populations. The condition of the cells so treated will be assayed by morphological observation with phase contrast microscopy which will be correlated with cell viability

1003545788

Source: https://www.industrydocuments.ucsf.edu/docs/fydm0000

as determined by the trypan blue dye exclusion test. Similar studies will be performed on alveolar macrophages obtained from animals treated with tobacco products in vivo. After exposure to tobacco products, the macrophages will be transferred to fresh media containing KLH bound to bentonite particles and following incubation for varying periods of time, the cells will be fixed in glutaraldehyde and the number of vacuoles in the cytoplasm will be enumerated and compared to untreated controls.

Once that information is obtained, treated macrophage populations will be incubated with ^{125}I-labeled KLH and the uptake, fate and localization of this material will be evaluated as described. In these studies we will employ the various physical forms of KLH. These studies should provide information as to the effect of tobacco products on the phagocytic functions of macrophages.

(2) Immune response

It has been shown that both free macrophage-processed antigen and antigen bound to macrophage membranes after processing can be immunogenic (17) both in vivo and in vitro. The mode of presentation of antigen appears to be a function of the chemical and physical nature of the antigen.

(1) Primary immune response in vivo

In one series of experiments, macrophages will be exposed to antigen before they are treated with cigarette smoke, while in another series, the cells will be treated with the cigarette smoke before allowed to react with antigen. After various periods of incubation, the macrophages will be collected, washed to remove free antigen, and injected into nonimmune syngeneic animals. Circulating levels of antibody will be measured by the described procedures after collection of serum on various days following primary immunization. Comparison will be made with control animals which will be injected with antigen-treated macrophages which were not exposed to cigarette smoke. Similar experiments will be carried out with alveolar macrophages obtained from animals exposed to cigarette smoke in vivo.

(2) Primary immune response in vitro

As stated previously we have been working as part of the Pulmonary Program Project on an in vitro model of antibody formation patterned after the Mishell-Dutton system in which we are studying the immunologic functions of alveolar macrophages. A likely extension of this work would be to determine the effect of cigarette smoke on the ability of lymphoid organs derived from smoked-animals to participate in such a response and further to determine if macrophages from smoked-animals could restore immunological function in macrophage-depleted cultures.

(3) Secondary immune response in vivo

Animals will be injected with a primary dose of KLH in vivo. Macrophages from nonimmune animals will be exposed to cigarette smoke and antigen as described above and then injected into the primed animals. The levels of circulating antibody will be measured one week after the animals are given the secondary injection. To determine whether macrophage-associated antigen can prime animals for a secondary response, we will inject macrophages incubated with antigen either with or without treatment by cigarette smoke and then challenge with free KLH. Serum will again be collected and assayed for antibody levels.

1003545789

Source: https://www.industrydocuments.ucsf.edu/docs/nyom0000

(4) Secondary immune response in vitro

Lymph nodes and spleens from primed animals will be removed and macrophages will be separated from the populations by standard techniques. Macrophages from smoked-exposed and control animals will be added to the lymphocyte cultures. Antibody formation will be determined by the plaque assay.

2. Effect on B and T cells

Using the reagents previous discussed (ie AMG and APCS) along with the congenitally athymic mouse system we can obtain cell populations in a relatively "pure" state to study individual cell functions. In these studies we will determine the effect of cigarette smoke on mitogenic responses. Protocols similar to those described in section 3a will be employed. The nude mouse system will also enable us to test our hypothesis that T-independent antigens are also macrophage-independent. The reasoning goes as follows: if we find that the macrophage is the prime target of the immunosuppressive effect of cigarette smoke and if our hypothesis is correct, there should be no immunosuppression observed in animals exposed to smoke and injected with a T-independent antigen. If an immunosuppressive effect is associated with the B cell, then cigarette smoke should suppress the response. By using combinations of T-dependent and T-independent antigens in nude and conventional mice we should provide further information as to the particular cells affected by cigarette smoke.

e. Effect of cigarette smoke on developmental aspects of the immune response

As stated previously, there is evidence that the immunological immaturity in the newborn of the species is not due to the lack of antibody-synthesizing cells but may primarily be due to the lack of antigen recognition or antigen processing cells in the form of competent macrophages. The studies proposed in this section will be concerned with the effects of cigarette smoke on the maturation of the immune response.

(1) Effect of cigarette smoke treatment of pregnant mice

Groups of pregnant mice will be exposed to cigarette smoke according to various protocols as described in section 3-a above. Similar groups of pregnant mice will be subjected to the stress effects of the smoking machine in the absence of cigarettes. In our basic design, pregnant mice will be exposed to smoke for one, two or three weeks of the gestation period. The time of smoke initiation will be varied, ie, the first week, the last week, the middle period etc. Litters will be assessed for immunological function at various times after birth; during early stages up to adult life. Litters will also be assessed for birth weight, litter size, and presence of deformed or defective animals. In another series of experiments litters will be divided in half, one half will receive further exposure to cigarette smoke while the others will remain untreated. Comparisons will be made with litters from sham-smoked animals. In order to determine if there are any pronounced physiological differences due to increased numbers of pregnancies, we will keep records of the number of times each female is mated and compare similar parameters of immunological function in succeeding litters from the same mother.

(2) Effect of cigarette smoke on mice of different ages

Animals will be exposed to cigarette smoke at regular intervals starting immediately after birth. Appropriate adjustments will be made for dose of cigarette smoke for neonatal animals. The animals will then be assessed for immunological function at varying time periods thereafter.

1003545790

Source: https://www.industrydocuments.ucsf.edu/docs/nyam0000

(3) Effect of cigarette smoke on the ability of macrophages to potentiate neonatal responses

Bendinelli et al. (84) have shown that peritoneal cells were capable of potentiating the immune response of neonatal animals to SRBC. We will design similar experiments using macrophages from smoked-animals of different ages as a source of the potentiating cells. We will also use normal macrophages in smoked animals to determine if they can restore immune function.

The studies of the effects of cigarette smoke on immune function described in preceeding sections can certainly be applied to a developmental approach in that they be carried out with animals of different age.

f. Effect of cigarette smoke on lung pathology

A collaborative effect has been established with Dr. M. Robinowitz in the Department of Pathology. The following section represents his input and interests in the project.

The pathologic examination of the mice used in the above experiments will be performed to assess the presence of intercurrent infectious processes especially mycoplasma pneumonia (85). The lungs will be removed from animals involved in the various smoke exposure protocols and will be fixed by perfusion with 10% buffered formalin solution by pressure fixation (86-87). The fixed lungs will be cut sagitally so that airways can be located. Sections will be taken from the entire right and left lungs of each animal. These sections will be evaluated for the overall pulmonary architecture with special emphasis on small airway changes. The bronchi will be examined for changes in mucus glands, mucosa, and all areas will be examined for inflammatory cell numbers, distribution and pigmentation using H and E, PAS, alcian blue and trichrome stains (50,88-91). The results of the pools of controls, sham controls and smoked-animals will be tabulated and examined with a code which will not be broken until after completion of the study by one individual (M.R.). The data will be correlated with the degree and duration of smoke exposure and the status of the immune response (50).

D. Collaboration

As indicated above, a collaborative effort has been established with Dr. M. Robinowitz. This will aid us in evaluating our data in view of potential intercurrent infection of our experimental animals. Dr. Robinowitz has begun some preliminary work on lungs from our animals. He has found that from 5-10% of the animals in each of the groups (smoked, sham-smoked and bench controls) had pneumonia. Identification of these animals had removed them from our protocols. In addition, he has found, in only one case, the presence of what appeared to be a squamous cell carcinoma of the lung. At this point, the significance of this finding remains unclear. Certainly, Dr. Robinowitz' input will be an addition to this project in terms of identifying infection and may provide further insight into pathological changes in experimental animals.

Another area of collaboration that is anticipated is with Dr. P. Hamosh and his group. There are obvious areas in both of our protocols where there is common interest. Both of us are proposing studies of the effect of cigarette smoke on maturational aspects. Certainly there will be an opportunity for the sharing of common materials to be used in our immunological and their biochemical and physiological studies. Such overlaps should generate a forum for exchange of scientific thinking as well.

1003545791

Source: https://www.industrydocuments.ucsf.edu/docs/rydm0000

F. Significance of the Proposed Research

It is well appreciated that the normal functions of the immune system protect the individual from constant challenge by foreign materials. A breakdown of immune function, whether under natural circumstances (immunological deficiency disease and aging) or induced (treatment with immunosuppressive agents) or due to environmental exposure (cigarette smoke) renders the individual subject to infection by microorganisms or prone to the development of various forms of cancer.

A potentially essential link in the chain of events leading to the initiation of both the humoral and cellular immune response is the interaction of antigen with macrophages. It is suggested that this interaction results in the processing of antigen thus rendering it immunogenic. Treatment of macrophages with various agents known to interfere with their phagocytic function results in a suppressed immune response. The proposed research should generate information which may provide insight into the possible means by which cigarette smoke may affect immune function. Since alveolar macrophages play an important role in the maintenance of lung stability, the proposed research is of direct significance to the understanding of the relationship between tobacco smoke and chronic lung disease. Further, the results of these studies should provide information which is currently unavailable concerning the effects of cigarette smoke on the maturation of the immune response.

In addition, through the proposed research, we hope to elucidate the mechanism of antigen recognition. If we can determine which physical forms of antigen are processed by macrophages, we may acquire a better understanding of why certain antigens lead to a breakdown of immune surveillance (immunological tolerance) whereas others initiate the immune response.

1003545792

Source: https://www.industrydocuments.ucsf.edu/docs/nydm0000

References

1. Niewoehner, D.E., Kleinerman, J., and Rice, D.B. New Eng. J. Med. 291: 755, 1974.

2. Auerbach, O., Stout, A.P., Hammond, E.C., and Garfinkel, L. New Eng. J. Med. 265:253, 1961.

3. Johnson, J.E. and Waldman, R.H. Transactions of Am. Clin. and Climat. Assoc. 84:237, 1972.

4. Waldman, R.H. and Henney, C.S. J. Exp. Med. 134:2, 1971.

5. Waldman, R.H., Spencer, C.S., and Johnson, J.E. Cell. Immunol. 3:294, 1972.

6. Jurgensen, P.F., Olsen, G.N., Swenson, E.W., Johnson, J.E., Henney, C.S., and Waldman, R.H. Am. Rev. Resp. Diseases 105:995, 1972.

7. Nettesheim, P. and Williams, M.L. Ann. N.Y. Acad. Sci. 221:220, 1974.

8. Green, G.M. Med. Clin. North Amer. 57:547, 1973.

9. Creasia, D.A., Nettesheim, P., and Hammons, A.S. Arch. Environ. Health 26:197, 1973.

10. Hiramoto, R.N. and Hamlin, N.M. Proc. Soc. Exp. Biol. Med. 134:996, 1970.

11. Holub, M. and Hauser, R.E. Immunol. 17:208, 1969.

12. Green, G.M. and Kass, E.H. J. Exp. Med. 119:167, 1964.

13. Allison, A.C. Ann. N.Y. Acad. Sci. 221:299, 1974.

14. Green, G.M. Med. Clin. N. Amer. 57:547, 1973.

15. Katz, D.H. and Benecerraf, B. Adv. Immunol. 15:1, 1972.

16. Silverman, M.S. J. Retic. Soc. 8:105, 1970.

17. Unanue, E.R. Adv. Immunol. 15:95, 1972.

18. Schwartz, R.S., Ryder, R.J.W., and Gottlieb, A.A. Prog. Allergy 14:81, 1970.

19. Gottlieb, A.A. and Waldman, S.R. In Macrophages and Cellular Immunity, CRC Press, Cleveland, 1972.

20. Holter, H. Int. Rev. Cytol. 8:481, 1959.

21. Heroch, J.F. J. Exp. Med. 116:827, 1962.

1003545793

Source: https://www.industrydocuments.ucsf.edu/docs/nyam0000

22. Maruta, H. and Mizuno, D. Nature New Biology 234:246, 1971.

23. Cruchaud, A. and Unanue, E.R. J. Immunol. 107:1329, 1971.

24. Gallily, R. and Feldman, M. Immunology 12:197, 1967.

25. Sabet, T.Y. and Friedman, H. Immunology 19:843, 1970.

26. Panijel and Cayeux, P. Immunology 14:769, 1968.

27. Argyris, B.F. and Plotkin, D.H. J. Immunol. 103:372, 1969.

28. Feldmann, M. and Palmer, J. Immunology 21:685, 1971.

29. Mosier, D.E. Science 158:1575, 1967.

30. Mosier, D.E. and Coppleson, L.W. Proc. Nat. Acad. Sci. 61:542, 1968.

31. Feldmann, M. J. Exp. Med. 136:737, 1972.

32. Nelson, D.S. In Macrophages and Cellular Immunity, CRC Press, Cleveland, 1972.

33. Harris, J.O., Swenson, E.W., and Johnson, J.E. J. Clin. Invest. 49: 2086, 1970.

34. Holt, P.G. and Keast, D. Proc. Sco. Exp. Biol. Med. 142:1243, 1973.

35. Meyer, D.H. et al. Arch. Environ. Health 22:362, 1971.

36. Roque, A.L. and Pickren, J.W. Acta Cytol. 12:420, 1968.

37. Pratt et al. Lab. Invest. 24:331, 1971.

38. Leuchtenberger, C. and Leuchtenberger, R. Exp. Cell Res. 62:161, 1970.

39. Holt, P.G. and Keast, D. Arch. Environ. Health 26:300, 1973.

40. Green, G.M. and Carolin, D. N. Eng. J. Med. 276:421, 1967.

41. Eichel, B. and Shahrik, H.A. Science 166:1424, 1969.

42. Holt, P.G. and Keast, D. Life Sciences 12:377, 1973.

43. Finklea, J.F. et al. Amer. Rev. Resp. Dis. 104:368, 1971.

44. Roszman, T.L. and Rogers, A.S. Amer. Rev. Resp. Dis. 108:1158, 1973.

45. Thomas, W., Holt, P.G., and Keast, D. Nature 243:240, 1973.

46. Thomas, W., Holt, P.G. and Keast, D. Int. Arch. Allergy 46:481, 1974.

47. Esber, H.J. et al. Arch. Environ. Health 27:99, 1973.

1003545794

Source: https://www.industrydocuments.ucsf.edu/docs/xwwl0136

48. Thomas, W., Holt, P.G., and Keast, D. Nature 248:358, 1974.

49. Thomas, W., Holt, P.G., and Keast, D. Arch. Environ. Health 30:78, 1975.

50. Rylander, R. Arch. Environ. Helath 29:329, 1974.

51. Thomas, W., Holt, P.G., and Keast, D. Arch Environ. Health 27:372, 1973.

52. Suciu-Foca, N. et al. The Lancet, 1062, May, 1974.

53. Whitehead, R.H. et al. The Lancet, 1233, June, 1974.

54. Desplaces, A., Charreire, J., and Izard, C. Rev. Eur. Etud. Clin. Biol. 16:822, 1971.

55. Warr, G.A. and Martin, R.R. Infect. Immunity 8:222, 1973.

56. Roszman, T.L., Elliott, L.H., and Rogers, A.S. Amer. Rev. Resp. Dis. 111: 453, 1975.

57. Burnet, F.M. "The Clonal Selection Theory of Acquired Immunity." Vanderbilt Univ. Press, Nashville, 1959.

58. Good, R.A. and Finstead, J. J. Nat. Canc. Inst. Monograph 31:41, 1964.

59. Teller, M.N. et al. J. Nat. Canc. Inst. 33:649, 1964.

60. Malgrem, R.A., Bennison, B.E., and McKinley, T.W. Proc. Soc. Exp. Biol. Med. 79:484, 1952.

61. Argyris, B.F. J. Exp. Med. 128:459, 1968.

62. Medawar, P.B. and Woodruff, M.F.A. Immunology 1:27, 1958.

63. Argyris, B.F. Transplantation 8:241, 1969.

64. Braun, W. and Lasky, L.J. Fed. Proc. 26:642, 1967.

65. Myrvik, Q.N., Leake, E.S., and Fariss, B. J. Immunol. 86:128, 196.

66. Argyris, B.F. J. Immunol. 99:744, 1967.

67. Bennett, B. Amer. J. Pathol. 48:165, 1966.

68. Schoenberg, M.D. et al. J. Cell. Physiol. 71:133, 1968.

69. Herscowitz, H.B., Harold, W.W., and Stavitsky, A.B. Immunology 22:51, 1972.

70. Proctor, M.L. Ph.D. Thesis, Georgetown University, 1975.

71. Mishell, R.I. and Dutton, R.W. J. Exp. Med. 126:423, 1967.

72. Herscowitz, H.B. and Stavitsky, A.B. J. Immunol. 105:1389, 1970.

1003545795

Source: https://www.industrydocuments.ucsf.edu/docs/yjmf0000

73. Jerne, N.K. and Nordin, A.A. Science 140:405, 1963.

74. Cunningham, A.J. and Szenberg, A. Immunology 14:599, 1968.

75. Herscowitz, H.B., DiBlasio, R.C., and Rosenberg, J.B. J. Immunol. Meth. 6:331, 1974.

76. Self, C. et al. Immunochem. 11:227, 1974.

77. Herscowitz, H.B. and Stylos, W.A. Prostaglandin (submitted).

78. Peavy, D.L. et al. J. Immunol. 111:352, 1973.

79. Unanue, E.R. and Feldman, J.D. Cell. Immunol. 2:269, 1971.

80. Pelley, R.P., Schwartz, H.J., and Stavitsky, A.B. Fed. Proc. 32:988, 1973.

81. Rocklin, R.E., Meyers, O.L., and David, J.R. J. Immunol. 104:95, 1970.

82. Pelley, R.P. and Schwartz, H.J. Proc. Soc. Exp. Biol. Med. 141:373, 1972.

83. Cohn, Z.A. and Ehrenreich, B.A. J. Exp. Med. 129:201, 1969.

84. Bendinelli, M., Senesi, S., and Falcone, G. J. Immunol. 106:1681, 1971.

85. Simmons, M.L. and Brick, J.O. In The Laboratory Mouse, Prentice Hall, Inc., Englewood Cliffs, N.J., 1970.

86. Loosli, G.G., Hertweck, M.S., and Hockwald, R.S. Arch. Environ. Health 21: 332, 1970.

87. Kleinerman, J. and Cowdrey, C.R. Yale J. Biol. Med. 40:579, 1958.

88. Kilburn, K.H. et al. Chest 64:615, 1974.

89. Pratt, P.C. and Kilburn, K.H. Chest 59:395, 1971.

90. Kilburn, K.H. et al. Chest 67:545, 1975.

91. Mawdesley-Thomas, L.E. and Healey, P. Arch. Environ. Health 27:248, 1973.

1003545796

Source: https://www.industrydocuments.ucsf.edu/docs/xkdh0000